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1. Nat Struct Biol. 1996 Dec;3(12):1040-5.
2. Proteins 1996 Nov;26(3):358-60.
3. Adv Exp Med Biol. 1996;409:251-4.

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X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy

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The three-dimensional structure of the major birch pollen allergen, the 17,500 M_r acidic protein Bet v 1 (from the birch, *Betula verrucosa*), is presented as determined both in the crystalline state by X-ray diffraction and in solution by nuclear magnetic resonance (NMR) spectroscopy. This is the first experimentally determined structure of a clinically important inhalant major allergen, estimated to cause allergy in 5–10 million individuals worldwide. The structure shows three regions on the molecular surface predicted to harbour cross-reactive B-cell epitopes which provide a structural basis for the allergic symptoms that birch pollen allergic patients show when they encounter pollens from related trees such as hazel, alder and hornbeam. The structure also shows an unusual feature, a 30 Å-long forked cavity that penetrates the entire protein.

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Allergens are proteins characterized by their ability to induce a pathogenic IgE response in susceptible individuals[1,2], giving rise to manifestations of allergy such as asthma, rhinitis and atopic dermatitis[3,4]. Birch pollen constitutes a major source of airborne allergens during early spring in the temperate climate zone of the northern hemisphere. The Bet v 1 protein[5,6] from *B. verrucosa* is the major allergen in birch and is homologous to the major pollen allergens from taxonomically related trees[7] Aln g 1 (*Alnus glutinosa*, alder)[8], Cor a 1 (*Corylus avellana*, hazel)[9] and Car. b 1 (*Carpinus betulus*, hornbeam)[10] of the *Fagales* order. In addition to their role as highly potent allergens, these pollen proteins are strongly related to a large number of proteins belonging to the group I plant pathogenesis-related proteins (PR-proteins) as revealed by high amino acid sequence identity[11,12]. Although it is widely accepted that these latter proteins are engaged in the plants' defence against microbial attack, the exact mechanism of their biological activity is not clear[13]. Due to the high similarity both in sequence and size to the group I PR-proteins, it is likely that the *Fagales* pollen allergens have the same function and act by a similar type of molecular mechanism. Bet v 1 is rapidly exported from pollen upon rehydration and about 20 isoforms of Bet v 1 can be resolved by 2-D gel electrophoresis of birch pollen extracts. Analysis of pollens collected from individual birch trees has shown that each tree expresses a specific sub-set of these isoforms which may reflect a function for Bet v 1 either in protection or recognition during the process of fertilization.

The protein structure determination of the birch pollen allergen Bet v 1 is a prerequisite for the understanding of the molecular interactions between tree pollen allergens and the human immune system that evokes the immediate hypersensitivity reactions in allergic diseases. Examination of naturally occurring amino acid substitutions in the Bet v 1 family of homologous pollen allergens provides a structural basis for the clinically observed cross-reactivity of tree pollen allergic patients' serum IgE. The structure determination also provides a basis for the understanding of the biological function of Bet v 1 in pollen.

The structure

The structures of Bet v 1 were determined independently by the two methods, X-ray diffraction and NMR spectroscopy. The two structures are essentially identical in their outline (Fig. 1*a–c*). The X-ray structure is determined to 2.0 Å resolution and an *R*-factor of 0.199 (Table 1). The NMR bundle of 20 structures aligns the C, N, and Cα of the peptide backbone to an average structure with an r.m.s.d. of 1.0±0.1 Å (Table 2). An alignment of the peptide backbone of the X-ray structure with the bundle of 20 NMR structures has an r.m.s.d. for the peptide backbone of 1.6 ±0.2 Å.

The main feature of the structure is a seven stranded anti-parallel β-sheet that wraps around a 25 residue-long C-terminal α-helix. The β-sheet and the C-terminal part of the long helix are separated by two consecutive helices. The seven stranded anti-parallel β-sheet consists of five longer strands of residues 2→11(I), 121←111(VII), 97→107(VI), 89←79(V), 66→75(IV), and two shorter strands 57←52(III), and 40→44(II). The C-terminal α-helix, (α3), consists of residues 130–154 and the consecutive helices consist of residues 15–23, (α1), and 25–34, (α2), respectively. This topology is strikingly similar to the structure of the C-terminal domain IV of rabbit muscle phosphoglucomutase[14] however, there is no homology between the two sequences (Fig. 1*e*).

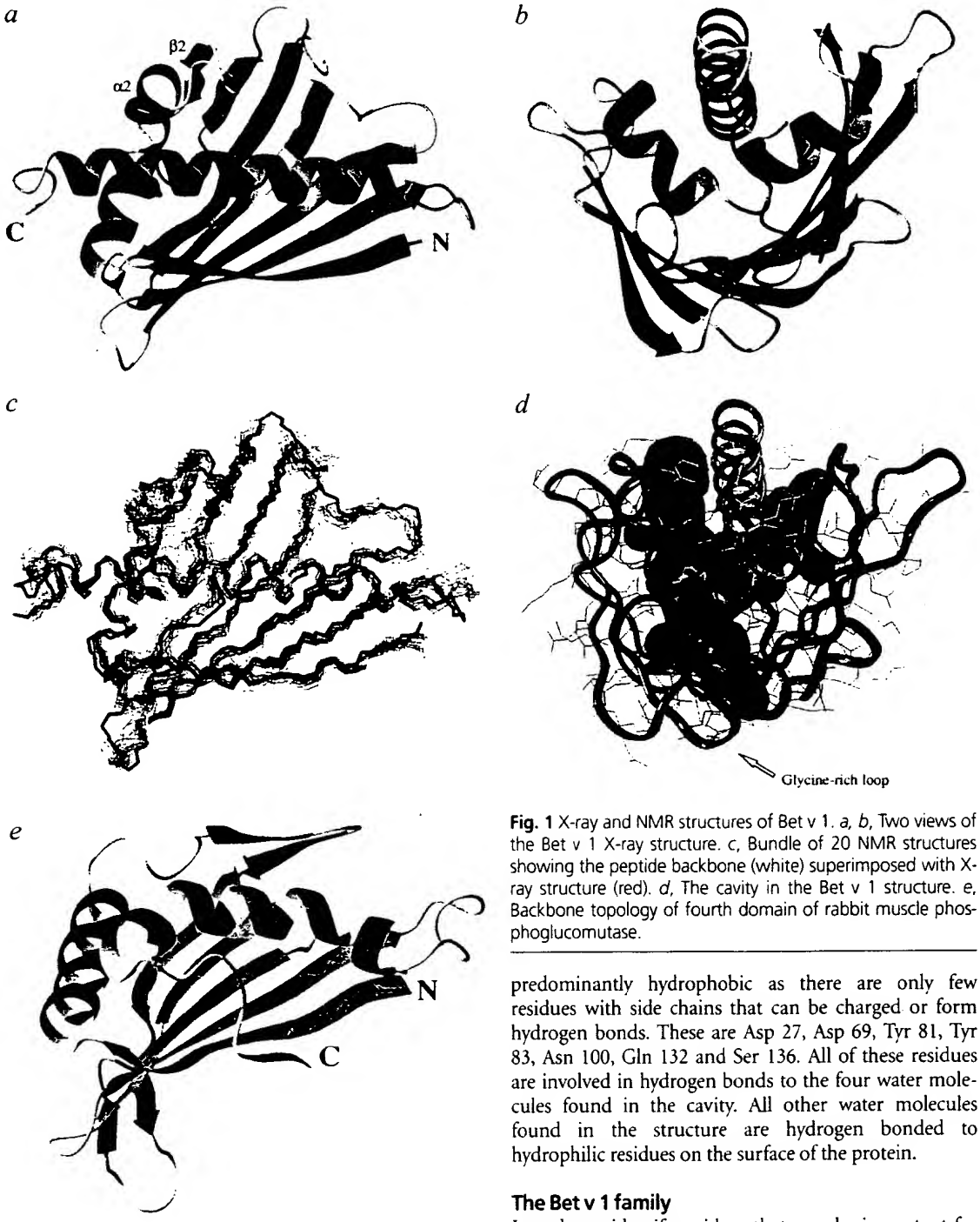


Fig. 1 X-ray and NMR structures of Bet v 1. *a, b,* Two views of the Bet v 1 X-ray structure. *c,* Bundle of 20 NMR structures showing the peptide backbone (white) superimposed with X-ray structure (red). *d,* The cavity in the Bet v 1 structure. *e,* Backbone topology of fourth domain of rabbit muscle phosphoglucomutase.

The hydrophobic core of Bet v 1 is formed by the C-terminal end of the α3 helix, the α1 and the α2 helices and hydrophobic residues in this vicinity from the β-strands β1, β5, β6 and β7. All three helices are clearly amphipathic with the hydrophobic residues clustering in this interior region.

Between the three helices and the β-sheet there is a large forked cavity that spans through the structure with three openings on the protein surface (Fig. 1d). The cavity is 30 Å long and has a volume of approximately 1500 Å^3. The presence of this cavity is a most unusual feature in a protein structure. The surface of the cavity is predominantly hydrophobic as there are only few residues with side chains that can be charged or form hydrogen bonds. These are Asp 27, Asp 69, Tyr 81, Tyr 83, Asn 100, Gln 132 and Ser 136. All of these residues are involved in hydrogen bonds to the four water molecules found in the cavity. All other water molecules found in the structure are hydrogen bonded to hydrophilic residues on the surface of the protein.

The Bet v 1 family

In order to identify residues that may be important for the biological function and structure of Bet v 1, 87 related amino acid sequences from other *Fagales* pollen allergens and PR-proteins were analysed with respect to conserved sequence positions and location in the structure (a complete list of all sequences and their alignment is available as supplementary material at *http://structbio.nature.com*). One particular feature revealed by this analysis (Fig. 2*a,b*) is the clustering of surface-exposed conserved polar residues in close vicinity to the cavity that runs through the Bet v 1 structure. Four of the conserved glycine residues, 46, 48, 49 and 51 form a glycine-rich loop connecting β-strands II and III. This sequence

Table 1 Summary of Bet v 1 structure determination

a, Data collection statistics for native and derivative crystals

	Native	PCMBS	$PtCl_4^{2-}$	oPt	$Au(CN)_2^-$
Resolution limit (Å)	2.0	3.0	2.4	2.0	2.8
R_{sym} (%)[1]	6.8	6.9	8.3	7.7	11.7
Unique reflections	9554	3030	5444	8169	3610
Observations	54458	12418	21007	31877	15221
Completeness (%)	95.8	98.5	92.9	82.8	89.3

b, MIR phasing statistics

Derivative	Phasing power[2]	R_{cullis}[2] (cen)	Res. (Å)	Occ.[3]	A. Occ.[3]	B(Å^2)[4]
PCMBS	1.65	0.63	3.0			
Site 1				0.42	0.28	34
Site 2				0.17	0.08	54
Site 3				0.13	0.02	56
Site 4				0.06	0.00	36
$PtCl_4^{2-}$	1.33	0.67	2.4			
Site 1				0.42	0.28	47
Site 2				0.22	0.13	56
Site 3				0.36	0.24	59
Site 4				0.20	0.15	40
Site 5				0.38	0.18	160
oPt	0.90	0.78	2.2			
Site 1				0.42	0.21	37
Site 2				0.33	0.22	96
Site 3				0.08	0.00	54
$Au(CN)_2^-$	0.80	0.78	2.8			
Site 1				0.16	0.14	36
Site 2				0.31	0.24	128
Site 3				0.11	0.11	93
Site 4				0.10	0.05	93

[1] $R_{sym} = \Sigma_{hkl}\Sigma_i|I(hkl)_i - \langle I(hkl)\rangle| / \Sigma_{hkl}\Sigma_i I(hkl)_i$.
[2] R_{cullis} and phasing powers are calculated using root mean squares (r.m.s.). E=lack of closure, E'=r.m.s.(E), R_{cullis}=r.m.s.$(E)/R_{ISO}$, phasing power=r.m.s.(f_H/E). $E=\Sigma||\boldsymbol{F}_{PH}\pm\boldsymbol{F}_P|-f_H|$, $E'=(E^2/n)^{0.5}$. R.m.s. $f(_H)=(\ \Sigma f_H^2/n)^{0.5}$, n=number of terms. $R_{ISO}=\Sigma_{hkl}|\ \boldsymbol{F}_{PH}-\boldsymbol{F}_P|\ /\ \Sigma_{hkl}\ \boldsymbol{F}_P$.
[3] Occ and A. Occ. are the refined occupancies and anomalous occupancies respectively.
[4] B is the thermal displacement parameter.

shows resemblance to the so-called P-loop motif (-G-X-G-G-X-G-) found in many nucleotide binding proteins[15] such as adenylate kinases and guanine nucleotide binding proteins. Several of the nucleotide binding proteins contain a conserved lysine residue that interacts with the phosphoryl group on the bound nucleotide[15] and it is interesting to note that the conserved Lys 54 in Bet v 1 is pointing towards the glycine-rich loop and could possibly serve a similar function. The position of the glycine-rich loop in the near proximity of one of the openings of the large cavity strongly suggests that these structural features are part of a site that is important for the biological function of the protein.

It has previously been suggested that Bet v 1 is a ribonuclease[16]. However, the exact specificity has not yet been demonstrated and no structural homology was found to any known RNAses in the Protein Data Bank in a search using the Dali method[17] for protein structure comparison.

Epitopes of Bet v 1

Human CD4+ T-cells have been shown to play a pivotal role in the pathophysiology of allergic disorders[18,19]. A number of T-cell epitopes have been identified in Bet v 1 by *in vitro* T-cell proliferation studies using overlapping synthetic peptides derived from the Bet v 1 sequence[20,21]. These identified T-cell epitope regions are distributed over almost the entire Bet v 1 structure, with no obvious preference for specific structural elements (not shown). This indicates that the processing of Bet v 1 by antigen-presenting cells and subsequent presentation of peptides to T-cell receptors in the context of HLA class II molecules is independent of the tertiary structure of Bet v 1.

It is interesting to note that one region, 48–59, of the Bet v 1 molecule appears not to contain T-cell epitopes. A cumulative total of 110 T cell clones and three polyclonal T cell lines derived from 25 different individuals has shown that the entire Bet v 1 molecule is immunogenic for allergic, as well as for non-allergic donors, except for this particular region[20–22]. Although the reason for this is not clear, this region includes the glycine-rich loop region which is highly conserved, not only among *Fagales* allergens and PR-proteins, but also among several human nucleotide-binding proteins[15]. Thus T-cells reacting with such a conserved epitope might cross-react with self-proteins and would therefore have been eliminated during the thymic selection of the T-cell repertoire.

Birch pollen allergic patients' serum IgE cross-react extensively with pollen allergens from other *Fagales* species. Both species-specific (birch) and cross-reactive (common for *Fagales* allergens) antibody binding epitopes have been demonstrated[23]. Antibodies raised by natural exposure interact primarily with their target antigens through conformational epitopes involving surface-exposed residues. Crystallographic studies of Fab–antigen complexes suggest that a surface area covering up to about 600–900 Å^2 may be directly involved in antibody binding[24]. An analysis of the known major pollen allergen sequences in combination with an analysis of the determined protein surface showed that there are three patches larger than 600 Å^2 of coherently connected conserved surface residues on the determined Bet v 1 structure (Fig. 3). The largest area consists of surface exposed residues from the two β-sheet strands VI and VII, the turn between them, and part of the N-terminal part of the long α-helix. The second area consists of nine conserved residues mainly from the C-terminal end of the consecutive helices and the exposed C-terminal part of the long helix. The third consists essentially of all the residues of loop 41–52. These regions are potential candidates for harbouring cross-reactive antibody binding epitopes of *Fagales* allergens.

Table 2 Structural statistics of the 20 NMR structures of Bet v 11

Distance restraints (all)[2]	1186
intraresidue	152
sequential (|i-j|=1)	343
medium range (1<|i-j| 5)	203
long range (|i-j|>5)	428
hydrogen bonds	60
Dihedral angle restraints (All)	35
ϕ	0
χ	35
Deviations from experimental derived restraints	
Distance restraints (Å)/number per structure	
0.1–0.2	13.85
0.2–0.3	2.65
0.3–0.4	0.2
0.4–0.5	0.1
r.m.s. deviation	0.018 (± 0.002)
hydrogen bonds >0.4	0
Dihedral angle restraints (°) /number per structure	
>5	0
r.m.s. deviation	0.45 (±0.06)
Deviations from idealized geometry	
Impropers (°)/number per structure	
>5	0
r.m.s. deviation	0.43 (±0.12)
Bonds (Å)/number per structure	
>0.05	0
r.m.s. deviation	0.0023 (±0.0002)
Angles (°)/number per structure	
>5	0.3
r.m.s. deviation	0.57 (± 0.14)
Energies (kcal mol^{-1})[3]	
NOE	30 (±6)
Dihedral angle restraint	0.4 (±0.1)
Bond	42.3 (±0.6)
Angle	372(±2)
Improper	122 (±2)
Repel	40.86 (±9.65)
van der Waals	−257 (±32)
Hydrogen bond restraint	11.79 (±3.18)
Hydrogen bond	−103 (±5)
R.m.s. deviations of atomic positions (Å)[4]	
backbone (Cα, N,C)	1.0±0.1
All heavy atoms	1.4±0.1

[1]The number of experimentally derived restraints used in the NMR structure calculations and average values per structure of the deviations between the 20 accepted structures and the experimental restraints or idealised geometry are shown.
[2]Number of non-redundant distance restraints.
[3]The energies were calculated using final force constants of: k_{NOE}=50 kcal mol^{-1} Å^{-2}, k_{cdih}=200 kcal mol^{-1} rad^{-2}, k_{bond}=1000 kcal mol^{-1} Å^{-2}, k_{angle}=500 kcal mol^{-1} rad^{-1}, $k_{improper}$=500 kcal mol^{-1} Å^{-2}, k_{repel}=4.0 kcal mol^{-1} Å^{-2}. The van der Waals energy and hydrogen bond energies were calculated using the X-PLOR switched Lennard–Jones van der Waals energy function and hydrogen bond function using the parmallh3x.pro parameter file, but they were not included in the target functions used in the simulated annealing procedure.
[4]R.m.s. deviations from an average structure of the 20 structures of Bet v 1.

The three-dimensional structure of Bet v 1 is crucial for establishing any correlation between the molecular structure and its role as a highly potent allergen and will be important for the future development of improved specific therapy for allergic disorders. Furthermore, the structure represents a step towards understanding the hitherto unknown biological function of these pollen proteins and related group I PR-proteins.

Methods

X-ray diffraction. Crystallization of recombinant Bet v 1 and collection of native data to a resolution of 2.0 Å, in spacegroup $C222_1$ with only one molecule in the asymmetric unit has been described previously[25]. The structure was solved by the method of multiple isomorphous replacement (MIR) using a total of four different derivatives. All experimental data were collected with a local Rigaku R-axis II imaging plate system, using monochromatized Cu K_α radiation from a rotating anode operated at 9 kW. Data collection statistics for all derivatives included are shown above. Minor changes (not exceeding 1.5%) in cell dimensions were observed for all of the derivatives. All data were processed with the DENZO and SCALEPACK[26] programs. Further data processing was done with the CCP4 program package[27]. The program AGROVATA was used for analysing the data and for the averaging of equivalent measurements. The resolution cut-off criterion for all data sets was the internal R-factor not exceeding 0.25 in the outermost resolution shell. The first derivative obtained was para-chloro mercury(II) benzene sulphonic acid, (PCMBS). This derivative has one well defined site and three minor sites. For the major site, the coordinates could be determined easily from the Harker sections. The three minor sites were located using the double difference electron density maps. The heavy atom positions in the three additional derivatives (oPt is chloro(2,2':6',2"-terpyridine) platinum(II) chloride) were determined using cross-Fourier technique. The calculation and refinement of phases were done with the MLPHARE program including all derivative data (isomorphous and anomalous) to their resolution limit. After refinement, the observed combined figure of merit was 0.61 to 3.0 Å resolution. However, the figure of merit quickly dropped off for data of higher resolution than 2.5 Å. This map was inspected using the graphics display and showed features that could easily be recognized as secondary structure. Subsequently density modification techniques were applied using the program DM, including histogram matching, solvent levelling and Sayre's equation resolution extension to further improve the quality of the map. MIR phases were used as starting phases to 2.5 Å and the phases were extended all the way to 2.0 Å. The model building was initiated using this map. The calculated electron density map clearly showed the large β-sheet and the long α-helix. The electron density fitting was performed with the program O[28]. A model for the entire molecule, except for a short region at residues 60–65, was build from this map. To construct this region phase combination of the phases after density modification and the partial model phases was undertaken using the program SIGMAA.

The structure was refined using the program X-PLOR[29] applying the standard simulated annealing slow cooling protocol using all data in the range 40–2 Å. Bulk solvent correction was applied using a scale factor of 0.42 and a temperature factor of 110 Å^2. A test set consisting of 10% of the data was excluded from the refinements for cross-validation purposes. The weighting factor between empirical energy terms and the crystallographic residual term applied was 1/2 times that derived from the standard energy-gradient based technique. The final *R*-value obtained was 0.199 and the corresponding R_{free} was 0.259. The average deviations in bond lengths and angles were 0.006 Å and 1.3°, respectively. The Ramanchandran plot showed no residues in generously allowed regions and one (Asp 93 with good electron density) in disallowed regions. Pro 63 was found to crystallise in two trans conformations (each with the two preferred ψ-values), consequently two conformations of the chain were included in the region Asp 60–Lys 65; both with refined occupancies. The description of the electron density in this region is still not perfect, probably due to additional disorder caused by Leu 62 pointing into the solvent in one of the conformations. A part of the final $2F_o$–F_c electron density is shown in Fig. 4.

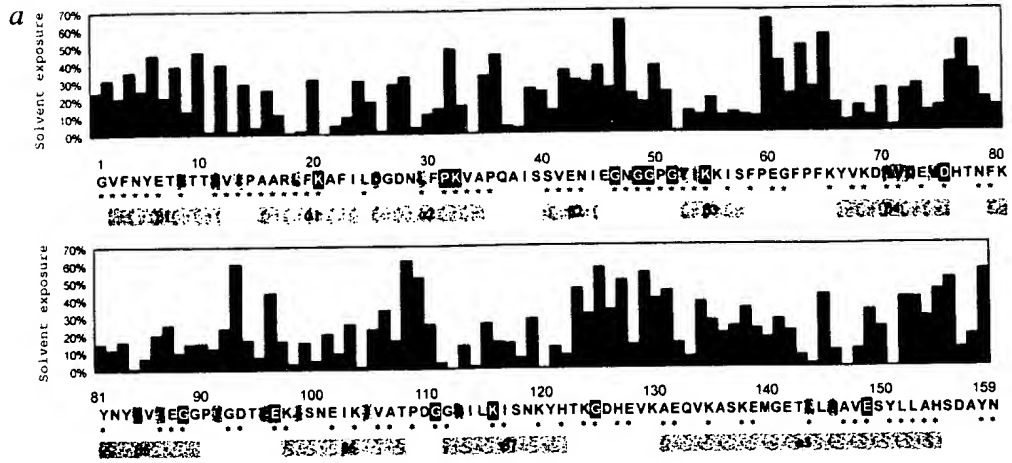
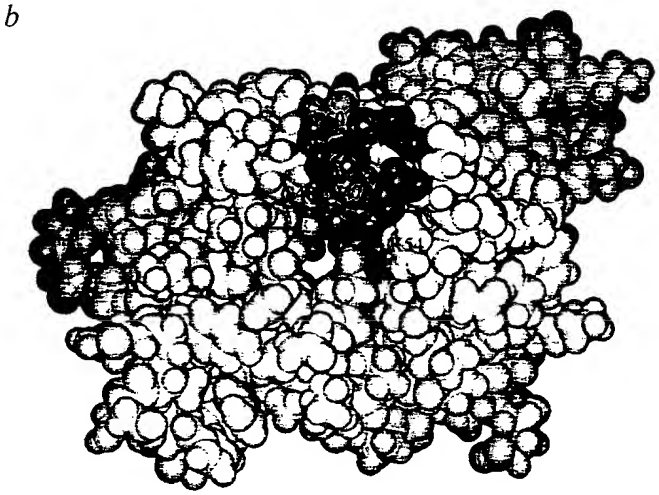


Fig. 2 Amino acid sequence of the structurally determined Bet v 1, solvent exposure of individual residues, secondary structure and comparison of 87 related protein sequences derived from cDNA sequences of *Fagales* allergens, group I pathogenesis-related and *Malus domestica* (apple) proteins. *a*, The residues which are completely conserved in ≥95% of the aligned sequences are shown in white letters on black, and those only mutated conservatively in ≥95% are shown in black letters on grey. Residues which are identical within the group of *Fagales* allergens are marked with asterisks. The exposure of each residue in the Bet v 1 structure is shown as a percentage relative to the free amino acid. *b*, View of the entrance of the cavity bordered by the glycine-rich loop and Lys 54.



A total of 85 water molecules were included in the model. Peaks were chosen automatically from the F_o–F_c electron density map using program XPLOR, based on having electron density greater than 4σ and being in hydrogen bonding distance from another hydrogen bond donor or acceptor. The peaks were inspected afterwards and only included if they showed a reasonable spherical shape and returned $2F_o$–F_c electron density after inclusion in the model.

NMR spectroscopy. Structure determination of recombinant Bet v 1 in solution by NMR spectroscopy was based on NMR spectra recorded of ^{13}C, ^{15}N double labelled, ^{15}N labelled, and unlabelled protein samples. Unlabelled recombinant Bet v 1 was expressed in *Escherichia coli* and purified as described[30]. For isotope labelling, *E. coli* cells were grown in defined media in the presence of ^{13}C-β-D-glucose and/or $(^{15}NH_4)_2SO_4$ and labelled Bet v 1 was purified as described[30]. All NMR spectra were recorded at 600 MHz using a Bruker AMX NMR spectrometer. Standard phase sensitive double quantum filtered COSY, TOCSY and NOESY (for references to the NMR experiments applied here see ref. 31) were obtained from the unlabelled samples at a concentration of 4.0 mM in 0.5 mM phosphate at pH 6.5 and 303 K. At the same condition but at 0.4 mM three-dimensional ^{15}N HMQC TOCSY and NOESY spectra were recorded of the ^{15}N-labelled sample, and triple resonance HNCACO and HNCA spectra were recorded of the double labelled sample at 0.4 mM. HCCH TOCSY spectra were recorded of the double labelled sample dissolved in deuterium oxide. The structure determination is based on the sequence specific assignments. These were obtained almost exclusively from ^1H NMR spectra and subsequently confirmed by sequential assignments of the heteronuclear NMR spectra. The structures were generated with a distance geometry/simulated annealing protocol using the X-PLOR version 3.1 computer program. The structure calculation used distance and dihedral angle restraints obtained for a total of 1131 NOEs with upper bounds of 2.7, 3.3, and 5.0 Å increased by 0.5 Å when the NOE restraint included a methyl group. For 34 residues,

Fig. 3 Front and back views of surface structures of conserved patches on the water-accessible surface of Bet v 1. The three coloured areas represent coherent stretches of the surface which are common to all Bet v 1 homologous tree pollen allergens sequenced so far and which cover at least 500 Å^2. The patches are comprised by backbone atoms and conserved side chains and represent potential highly cross-reactive B-cell epitopes on the protein surface. The area shown in orange consists of the β-strands (VI) and (VII), the turn between them, and part of the N terminus of the long α-helix. The second area in green consists of nine residues mainly from the second short helix and the C terminus of the long helix. The third area shown in pink includes the residues of the loop 41–52.

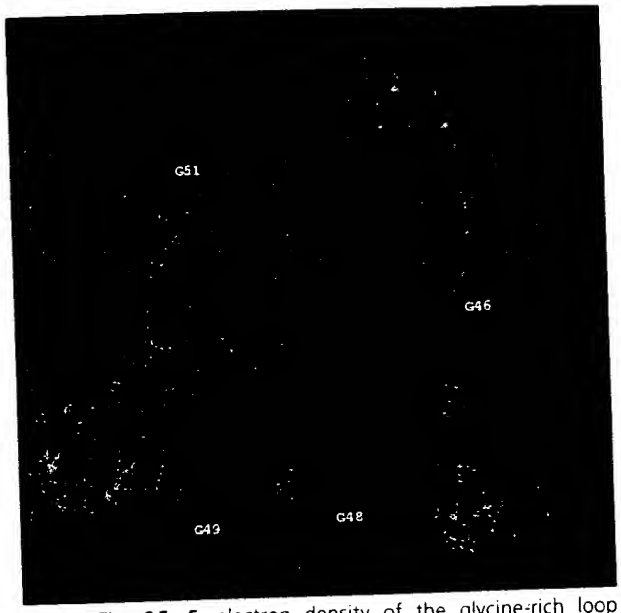


Fig. 4 The $2F_o$–F_c electron density of the glycine-rich loop 45–52. The electron density has been displayed using the mapcover option of the program O[28]. The map is contoured at the 1 σ level.

χ^1 dihedral angles were determined based on estimates of the $^3J_{H\alpha H\beta}$ coupling constants in the DQF-COSY spectrum and the relevant NOE intensities. Finally, 60 hydrogen bond restraints were selected and applied after inspection of the calculated hydrogen bond energies and/or evidence of slow amide hydrogen exchange.

Calculation of cavity. The dimensions of the cavity present in the Bet v 1 structure were calculated by the computer program SURFNET[32] using a probe size of 1.4 Å.

Sequence comparisons. Sequences from 56 *Fagales* group 1 allergens, three apple group 1 allergens and 28 pathogenesis-related proteins were used for alignment. Gaps introduced in some of the sequences during alignment were all considered as non-identical amino acid substitutions. The sequences were aligned and derived from GenPept release 93 using the score table blosum 62[33], apart from eight Bet v 1 sequences which were kindly provided by Dr. Yvan Boutin (personal communication). Protein sequences and alignment are available upon request to corresponding author. Requests for materials should be addressed to M.D.S (e-mail: *michaels@inet.uni-c.dk*), or to M.G. concerning X-ray data (e-mail: *michael@x-ray.ki.ku.dk*) or to F.M.P. (e-mail: *carlfmp@unidhp.uni-c.dk*) regarding NMR data.

Coordinates for the X-ray and NMR structures of Bet v 1 will be deposited in the Brookhaven Protein Data Bank.

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